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Ubiquitination and deubiquitination of PCNA in response to stalling of the replication fork

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Abbreviations: HU, hydroxyurea; MMS, methyl methanesulfonate; TLS, translesion synthesis; Ub-PCNA, mono-ubiquitinated PCNA; UV, ultraviolet light; XP-V, xeroderma pigmentosum variant.

Abstract

Following exposure of human cells to DNA damaging agents that block the progress of the replication fork, mono-ubiquitination of PCNA mediates the switch from replicative DNA polymerases to polymerases specialised for translesion synthesis. We have shown that this modification of PCNA is necessary for the survival of cells after UV-irradiation and methyl methanesulfonate, that it is independent of cell cycle checkpoint activation, and that it persists after UV damage has been removed. In this Extra-view, we compare the regulation and biological significance of PCNA ubiquitination following treatments with UV light and the replication inhibitor hydroxyurea. We show that ubiquitination persists after removal of the replication block in both cases. With UV however, the persistence of ubiquitinated PCNA correlates with disappearance of the PCNA deubiquitinating enzyme USP1, whereas this is not the case for HU. Prevention of PCNA ubiquitination sensitises the cells to killing by both UV and HU.

Introduction

All cells are continuously exposed to agents that damage DNA. Although much of the damage is repaired rapidly and efficiently, a significant proportion remains unrepaired during DNA replication and blocks the passage of the replication fork. In order to circumvent this damage, the cell must either employ a damage avoidance mechanism or replicate past the damaged base(s). Since the replicative polymerases are blocked by most types of damage, they have to be substituted with specialised low fidelity polymerases that are able to synthesise DNA past different lesions in a process known as translesion synthesis (TLS). The replacement of the replicative polymerase with the specialised TLS polymerase is known as a polymerase switch [1]. A central player in this polymerase switch, and indeed in almost all processes involving DNA polymerases, is the sliding clamp, PCNA. PCNA is a homotrimeric ring structure that encircles the DNA, tethers the polymerase to the DNA and thereby increases its processivity. A wealth of evidence has shown that a key step in this switching process is the mono-ubiquitination of PCNA [2-4]. In all eukaryotes studied, mono-ubiquitination of PCNA on lysine-164 is brought about by Rad6 and Rad18 orthologues [2, 4]. In *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* further ubiquitins are added by Ubc13-Mms2 and Rad5 (*S. cerevisiae*) [2] or its orthologue Rad8 (*S. pombe)* [5]. Poly-ubiquitination of PCNA is much harder to detect in mammalian systems, but there is increasing evidence to suggest that it does indeed occur [6, 7]. Most of the TLS polymerases belong to the Y-family and they all have ubiquitin–binding motifs in their C-terminal domains [8], and with one exception, they also have PCNA-binding (PIP) motifs. This provides a mechanism with which they can be recruited to Ub-PCNA at stalled forks.

The trigger for PCNA ubiquitination and its biological significance

Given the central importance of PCNA ubiquitination, a crucial question to be addressed is how the ubiquitination process is regulated. In particular, what is the trigger, what regulates the ubiquitination, and how and when does the ubiquitin get taken off the PCNA. We have addressed these questions in a recently published paper [9] and our results, together with those of others, are beginning to shed light on what appears to be a complex control mechanism. There is a consensus from several groups that the trigger is single-stranded DNA exposed in the vicinity of the blocked replication fork. This conclusion has been arrived at by comparison of ubiquitination of PCNA in response to different DNA damaging agents. Those that generate lesions that block fork progression cause PCNA ubiquitination, whereas those that generate double-strand breaks directly or indirectly do not. Furthermore agents that do not damage the DNA but slow down fork progression by depleting the cell of nucleotides (eg hydroxyurea (HU)) or by inhibiting replicative polymerases (eg aphidicolin) also trigger PCNA ubiquitination. These agents are thought to expose single-stranded DNA by allowing unwinding of the DNA ahead of the fork even when fork progression has been prevented. Single-stranded DNA inside all cells is coated with the single-strand DNA binding protein RPA. Rad18, the E3 ubiquitin ligase that catalyses mono-ubiquitination of PCNA, has been known for 20 years to bind single-stranded DNA [10] but recently Davies et al have shown that Rad18 also physically interacts with RPA [11]. So we have a satisfying coherent mechanism: blocked forks generate single-stranded DNA that gets coated with RPA. Rad18 is recruited, bringing its cognate E2 ubiquitin-conjugating partner Rad6 and they effect the mono-ubiquitination of PCNA. The ubiquitinated PCNA attracts TLS polymerases via their

ubiquitin-binding motifs and the polymerase switch is facilitated. Much of this process has been reconstituted recently in vitro [12].

RPA-coated single-stranded DNA is also known to be the trigger for activation of cell-cycle checkpoints via the ATR-ATRIP protein kinase [13]. Surprisingly however these two processes, checkpoint activation and PCNA ubiquitination, appear to be triggered independently. Evidence to support this contention has come from work with *S. cerevisiae* [11], *S. pombe* [5], Xenopus [14] and human cells [9], all coming to the same conclusion.

Many processes are activated inside the cell by DNA damage. Activation does not per se mean that it is necessarily of biological importance. To examine this in the case of PCNA ubiquitination, we were able, using siRNA, to generate cell lines that expressed almost exclusively exogenous PCNA that was either wild-type, or mutated at lysine 164 such that it could not be ubiquitinated. The cell line expressing mutant PCNA was sensitive to killing by ultraviolet (UV) light and methyl methanesulfonate (MMS), which both generate replication-stalling lesions, but not to bleomycin or camptothecin, which result in double-strand breaks in cells. This proved that PCNA ubiquitination was indeed of biological importance in protecting cells against the former type of lesion.

Persistence of PCNA mono-ubiquitination after UV or HU treament

We were next interested in understanding how the cell removed ubiquitin from PCNA after the damage had gone. A clue to this had come from the discovery that the de-ubiquitinating enzyme USP1 was able to remove ubiquitin from Ub-PCNA, and that

depletion of USP1 resulted in a significant level of Ub-PCNA even in undamaged cells[15]. To examine what happened in damaged cells we employed a couple of tricks. We used a cell line expressing two photolyases, enzymes which, when exposed to visible light, are capable of reversing the two major forms of UV damage in situ. We exposed these cells to UV, incubated them for a few hours to generate Ub-PCNA and then shone visible light on them to reverse the damage. We were surprised to find that despite removal of >90% of the damage, the PCNA remained ubiquitinated for several hours. Our next thought was that the PCNA was released from the chromatin into the nucleoplasm, where it would get diluted by the pool of unmodified PCNA. This however turned out not to be the case. The modified PCNA remained associated with the chromatin in the triton-insoluble fraction of the cell.

In view of the unexpectedness of these findings we felt it was important to confirm them using an independent method. We therefore exposed the cells to HU for 24h to inhibit replication and generate Ub-PCNA. We then removed the HU and released the cells into cycle. A 24-hour treatment with HU resulted in the generation of a substantial amount of Ub-PCNA (Figure 1A, lane 5), comparable to that produced after UV-irradiation (Figure 1A, lane 1). We then removed the HU to allow cells to continue to progress through the cell cycle. The flow cytometry profile of Figure 1B shows that the cells blocked in early S by the HU treatment traversed through S phase in the first nine hours following release of the HU block and went through mitosis within 24 h. Figures 1C and D show that DNA synthesis, measured by thymidine incorporation, and cell proliferation resumed after release of the block. These data show that after release from the HU block, cells traversed through the cell cycle and retained their proliferative capacity. Nevertheless, Ub-PCNA remained at relatively

constant levels for at least six hours after release of the block (Figure 1A, lanes 5-16). As before, the Ub-PCNA remained non-extractable by triton (Figure 1A, compare even and odd-numbered lanes). Although a small fraction, estimated at 10% of the population, appeared to remain blocked at the G1/S boundary several hours after removal of HU (Figure 1B, 6 and 9 h time-points), this cannot account for the maintenance of PCNA ubiquitination in the whole population (Fig 1A).

How can we reconcile the persistence of ubiquitinated PCNA with the existence of a DUB that de-ubiquitinates PCNA. A clue had already come from the D'Andrea lab, who showed that after high UV doses, USP1 disappeared from the cell [15]. They subsequently showed that USP1 normally turns over rapidly, and UV inhibits transcription of USP1 mRNA [16]. We confirmed their findings and showed that USP1 disappeared from the cell even after low UV doses, consistent with the persistence of PCNA ubiquitination after UV damage. However with other DNA damaging agents, the picture was less clear. After MMS treatment, an initial rise in PCNA ubiquitination corresponded with a drop in USP1 levels. However at later times, PCNA ubiquitination levels increased further even though USP1 levels were restored. Furthermore following HU treatment, there was no disappearance of USP1 under any circumstances (Fig 2A), even though PCNA ubiquitination persisted for several hours. A comparison of the responses of USP1 to doses of UV and HU that generate similar levels of PCNA ubiquitination is shown in Figure 2B. Whereas most of the USP1 disappeared after 10 Jm^{-2} UV (lane 4), there was no change in USP1 level after 5mM HU (lane 7). To explain this apparent inconsistency, we propose that following HU treatment, the USP1 is either sequestered away from the PCNA or is in an inactive form. Cohn et al have shown that USP1 interacts with a partner protein UAF1 and

this interaction is required for activity of USP1 [16]. It is possible that HU treatment results in disruption of this interaction.

These results that we have obtained with HU treatment are quite perplexing. We have proposed a model of TLS behind the replication fork to explain the persistence of Ub-PCNA after UV treatment. In this model, Ub-PCNA is left behind at the stalled fork and a new molecule of PCNA is used for replication restart beyond the lesion. This model is not however readily extendable to the situation with HU. We have shown, by DNA fibre labelling, that DNA synthesis continues from most stalled replication forks after removal of the HU block (SB and ARL, unpublished data). The persistence of Ub-PCNA suggests that either it is dislodged at the stalled fork before or at the time of reinitiation of synthesis, or synthesis reinitiates using the Ub-PCNA together with replicative polymerases. Ubiquitination of PCNA does not affect the activity of polδ in vitro [12, 17].

Function of PCNA ubiquitination following HU treatment

Although HU treatment of S phase cells indisputably triggers ubiquitination of PCNA, it is not obvious why this should have any functional significance. HU inhibits ribonucleotide reductase, resulting in a depletion of the deoxyribonucleotide pool, thereby stalling the progress of the replication fork. However HU does not generate DNA damage per se. Ubiquitination of PCNA will facilitate the recruitment of TLS polymerases to the blocked forks, but it is not clear how these polymerases could be of use if deoxyribonucleotides are unavailable and if there is no damage in the template strands. We therefore examined the sensitivity to HU of our PCNA-K164R expressing cell line. Figure 3 shows that the cells expressing mutant PCNA are indeed

sensitive to killing by HU, indicating that PCNA ubiquitination is important for maintaining the viability of cells depleted of deoxyribonucleotides.

What might be the role of Ub-PCNA in maintaining viability during HU treatment? The Ub-PCNA acts as a platform to facilitate recruitment of Y-family polymerases to the stalled fork, and consistent with our observations, Cleaver and colleagues reported that xeroderma pigmentosum variant (XP-V) cells deficient in TLS DNA polymerase η have abnormal responses to HU [18] and to other inhibitors of DNA replication [19]. In the presence of HU, more double-strand breaks as measured by γ-H2AX and Mre11 foci accumulated in XP-V than in normal cells [18]. In other studies, XP-V cells were found to be more sensitive than normal cells to nucleoside analogues 1-β-D-arabinofuranosylcytosine and gemcitabine, which, like HU, inhibit progression of the replication fork without damaging DNA[19]. Even in *Escherichia coli*, Y-family polymerases assist cells in surviving the effects of HU treatment [20].

In conclusion, although our understanding of PCNA mono-ubiquitination and factors affecting its regulation has substantially increased, especially following UV-irradiation, many questions remain to be answered about its role in response to other agents.

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Figure legends

Figure 1 Persistence of Ub-PCNA after HU treatment.

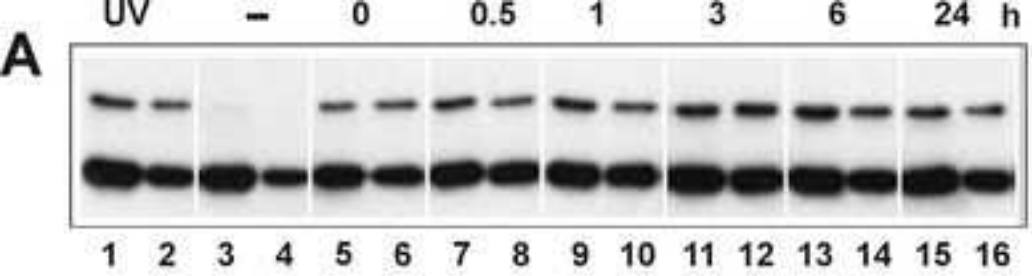
MRC5V1 cells were treated with 1 mM HU for 24 h. The cells were then washed in PBS and incubated further. At different times after release from the HU block, cells were analysed for (A) PCNA ubiquitination with (even lanes) or without (odd lanes) prior triton extraction (UV, 20 Jm^{-2} for 6h), (B) cell cycle progression by flow cytometry, (C) incorporation of ^{3}H thymidine into DNA and (D) cell number.

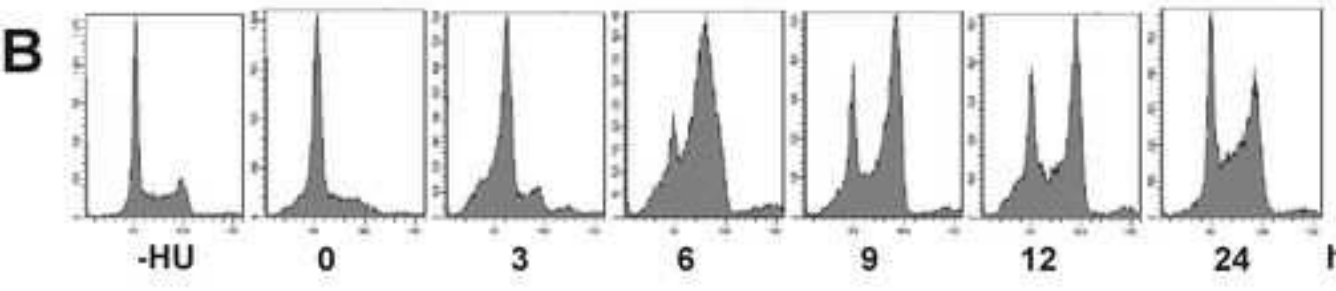
Figure 2 USP1 degradation and PCNA ubiquitination

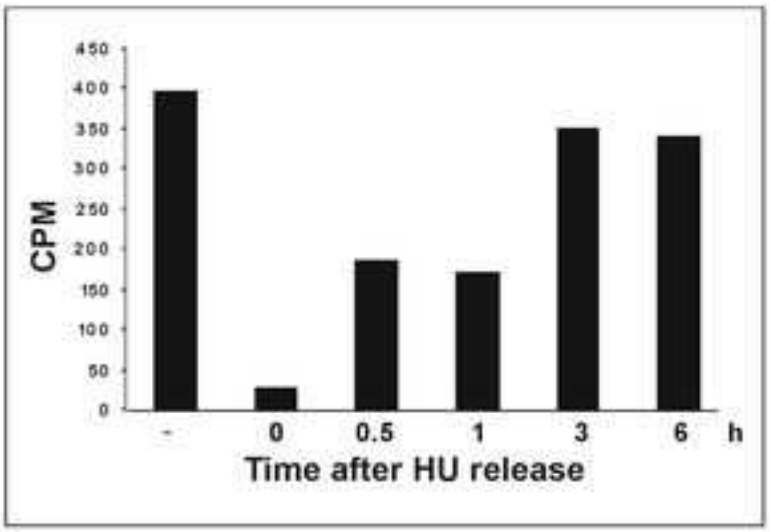
A, MRC5V1 cells were treated with 1mM HU for 24 h, washed twice in PBS and incubated for the indicated times. B, Cells were either untreated (lane 1) or UV-irradiated with the indicated doses, followed by incubation for 24 h (lanes 2-4) or treated with indicated doses of HU for 24 h (lanes 5-7). Lysates were analysed by immunoblotting with anti-USP1 (top panels) or anti-PCNA (bottom panels) antibodies.

Figure 3 HU sensitivity of cells expressing PCNA-K164R

MRC5V1 cell clones expressing his-tagged wild-type PCNA (WT) or PCNA-K164R (KR) were treated with PCNA-specific siRNA and subsequently incubated for 24 h with HU, prior to plating. Error bars: SEM of 5 experiments.


UV
–
0
0.5
1
3
6
24
h
A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16


B
-HU
0
3
6
9
12
24
h


C
CPM
450
400
350
300
250
200
150
100
50
0
-
0
0.5
1
3
6
h
Time after HU release

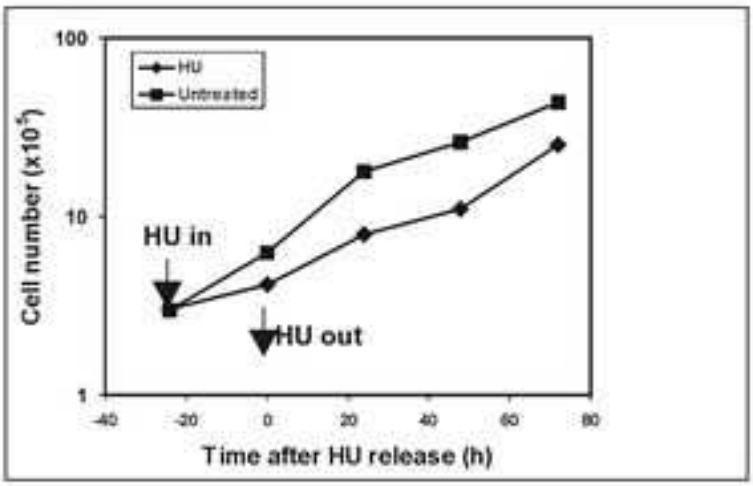

D
HU
Untreated
Cell number (x10⁵)
100
10
1
HU in
HU out
-40
-20
0
20
40
60
80
Time after HU release (h)

Fig 1

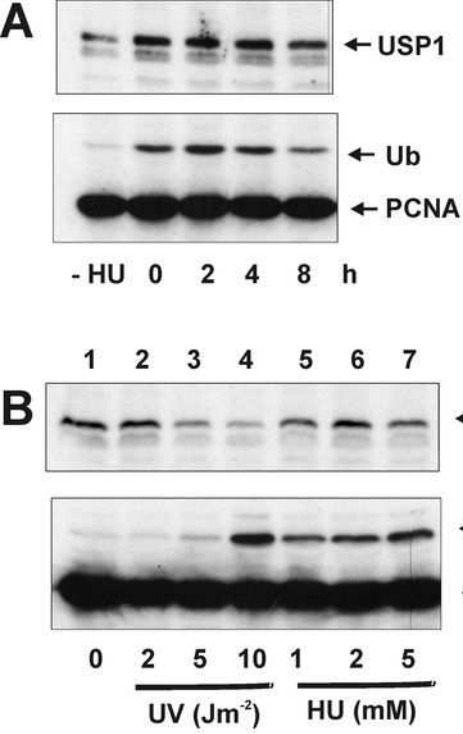

A
USP1
Ub
PCNA
- HU 0 2 4 8 h
1 2 3 4 5 6 7
B
USP1
Ub
PCNA
0 2 5 10 1 2 5
UV (Jm-2)
HU (mM)

Fig 2

Figure 3